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Detection of Resistance to Second-Line Antituberculosis Drugs by Use of the Genotype MTBDR*sl* Assay: a Multicenter Evaluation and Feasibility Study

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The rate of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) has been steadily increasing in countries of the former USSR. The availability of rapid and reliable methods for the detection of drug resistance to second-line drugs is vital for adequate patient management. We evaluated the performance of the Genotype MTBDR*sl* assay compared to that of phenotypic drug susceptibility testing (Becton Dickinson Bactec MGIT 960 system) with a test panel of 200 *Mycobacterium tuberculosis* isolates at four sites in Eastern Europe. The interpretability of the Genotype MTBDR*sl* assay was over 95%. The sensitivity for the detection of resistance to fluoroquinolones, ethambutol, amikacin, and capreomycin varied between 77.3% and 92.3%; however, it was much lower for kanamycin (42.7%). The sensitivity for the detection of XDR TB was 22.6%. The test specificity was over 82% for all drugs. The assay presents a good screening tool for the rapid detection of resistance to individual second-line drugs and can be recommended for use in countries with a high burden of MDR/XDR TB. The sensitivity for the detection of kanamycin resistance needs improvement.

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) has become a serious threat to global TB control due to difficulties in diagnosis and treatment, the high rates of treatment failure, the lower survival rates (especially in HIV-infected persons), and the associated high costs (25).

The Indian subcontinent, China, and Russia are believed to account for the majority of cases globally (23). The rate of MDR and XDR TB infections has been steadily increasing in countries of the former USSR (19) due to multiple incomplete treatment regimens and poor infection control practices against a rapidly evolving epidemic of HIV. Converging drug-resistant TB and HIV epidemics pose a serious problem and undermine the efforts of national TB control programs (5). There is growing evidence that drug resistance in these settings is strongly associated with the dominating Beijing family of strains (6, 10, 14, 15, 17, 21, 22).

In this situation, the availability of rapid, reliable, and accurate methods for the identification and detection of drug resistance in *M. tuberculosis* strains is vital for adequate patient management, leading to improved outcomes, a reduction of infectiousness, and the prevention of further nosocomial transmissions (8).

Automated liquid culture systems have significantly shortened turnaround times for drug susceptibility testing (DST) compared to the turnaround times with the use of solid media, but bacteriological assays are technically demanding and still take around 7 to 10 days from the point of the initial culture (18).

Line probe assays present a more rapid alternative (1). They have been extensively evaluated for the detection of resistance to isoniazid (INH) and rifampin (RIF) and have been recommended by the WHO for implementation on cultures and patient specimens globally (24). The recently developed Genotype MTBDR*sl* (Hain Lifescience GmbH, Nehren, Germany) assay for the detection of resistance to the main second-line drugs (SLDs) is potentially appealing for use in suspected or confirmed cases of MDR TB to screen for the presence of further resistance. The assay has been evaluated in low-incidence settings in two recent studies and showed good sensitivity and specificity (4, 7). However, larger studies in settings with a high TB burden and drug-resistant TB are required to demonstrate the feasibility of the use of the assay for the routine detection of XDR TB strains and also to evaluate its sensitivity and specificity, which could potentially be affected by the prevalence of certain types of mutations in regions (Russia and the Baltic States) where the Beijing family dominates.

We have conducted a multicenter, international study of diagnostic accuracy across four sites with the aim to assess the performance of the Genotype MTBDR*sl* assay in the detection of resistance to fluoroquinolones (FQs), aminoglycoside-capreomycin (AG-CP), and ethambutol (EMB) through the detection of key mutations associated with resistance to these drugs. In addition, the study was used to determine the abilities of multiple centers to perform a diagnostic assay correctly as part of the development of a clinical/diagnostic trial network in Eastern Europe within the European Union Seventh Framework Programme-funded project TB PAN-NET.

MATERIALS AND METHODS

The study was designed in accordance with standards for the reporting of diagnostic accuracy studies (STARD) principles of diagnostic accuracy studies (3).

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TABLE 1 Drug resistance patterns for the strain collection used in the study[a]

Group	No. of strains ($n = 200$)	Resistance pattern				
		INH	RIF	EMB	FQs	AG-CP[b]
Non-MDR TB strains ($n = 30$)	13	S	S	S	NA	NA
	1	S	S	S	S	S
	5	S	R	S	NA	NA
	1	R	S	S	NA	NA
	5	R	S	S	S	S
	1	R	S	S	S	R
	2	R	S	R	S	S
	1	R	S	R	S	R
	1	R	S	R	R	S
MDR TB strains ($n = 170$)	7	R	R	S	S	S
	32	R	R	R	S	S
	1	R	R	S	R	S
	4	R	R	S	S	R
	39	R	R	R	R	S
	46	R	R	R	S	R
	40	R	R	S, R	R	R
	1	R	R	R	NA	S

[a] MDR TB, multidrug-resistant tuberculosis; INH, isoniazid; RIF, rifampin; EMB, ethambutol; FQs, fluoroquinolones; AG/CP, aminoglycoside-capreomycin; S, sensitive; R, resistant; NA, not applicable.
[b] AG-CP indicates phenotypic resistance to any aminoglycoside and/or capreomycin.

TABLE 2 AG-CP resistance patterns[a]

No. of strains	Resistance pattern		
	AMK	KAN	CAP
89	S	S	S
2	S	S	R
42	S	R	S
1	R	S	S
1	S	R	R
9	R	R	S
1	R	S	R
35	R	R	R

[a] There was a total of 180 strains. One MDR strain had no phenotypic DST result for capreomycin (CAP). AMK, amikacin; KAN, kanamycin; S, sensitive; R, resistant.

Study material and bacteriological methods. A strain collection ($n = 200$) containing 170 MDR (85.0%) and 30 non-MDR (15.0%) TB strains was used; 40 MDR TB strains (23.5%) were XDR. The isolates were selected sequentially from the 2004-2010 archive of the Estonian National Tuberculosis Reference Laboratory, Tartu University Hospital, in quantities sufficient to meet predetermined statistical power calculation requirements (see below). All of the included isolates had been previously identified as *M. tuberculosis* isolates by the Accuprobe (Gen-Probe Inc.) or GenoType MTBC (Hain Lifescience GmbH) assay and had available first-line (all isolates) and second-line (all MDR and 11 non-MDR strains) DST results. The pattern of resistance of the collection is shown in Table 1.

Noticeably, 45/180 (25%) strains had different sensitivities to kanamycin and amikacin; of these strains, 43 were kanamycin resistant but amikacin sensitive, and 2 were kanamycin sensitive but amikacin resistant (Table 2).

DST was performed with the Bactec MGIT 960 system in the Tartu laboratory according to internationally accepted methods (2, 13), using the following concentrations of drugs: 1.0 μg/ml streptomycin (SM), 1.0 μg/ml INH, 1.0 μg/ml RIF, 5.0 μg/ml EMB, 100.0 μg/ml pyrazinamide (PZA), 2.0 μg/ml ofloxacin (Ofl), 1.0 μg/ml amikacin (AMK), 2.5 μg/ml capreomycin (CAP), and 5.0 μg/ml kanamycin (KAN) (13).

Archived isolates were subcultured on Lowenstein-Jensen (LJ) medium to obtain a viable culture in an amount sufficient for further DNA extraction.

DNA extraction and molecular methods. To prepare four identical study DNA panels to be further analyzed at the study sites (Brasov, Romania; Bucharest, Romania; Tartu, Estonia; and Samara, Russian Federation), crude DNA extracts were isolated from the cultures by heating (95°C for 30 min), followed by sonication (20 min). Each panel was relabeled in a unique way by an independent staff member to avoid assay interpretation biases. DNA samples had undergone quality control (QC) measures prior to dissemination to sites by using electrophoresis on a 1.5% agarose gel to demonstrate the presence of DNA of acceptable quality and quantity in the preparation. Unblinding of the results was performed after the completion of the experimental phase.

Two sites had no prior experience with the performance of the test; one site had moderate prior experience, while one site had extensive experience with the method.

One operator at each field site, blinded toward original phenotypic DST results, ran the Genotype MTBDR*sl* assay for all 200 samples and read results according to the manufacturer's instructions. All operators received standardized training on the method from the manufacturer prior to the study initiation in London, United Kingdom. Each site sent original result evaluation sheets with the assay strips attached to Samara, where two experienced operators, not involved in the original testing and blinded toward original phenotypic as well as well molecular assay results, reread all 800 membranes. All discrepant results were reviewed by a third independent experienced operator, and the majority opinion was taken as the final reference opinion.

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to assess the performance of the Genotype MTBDR*sl* assay. To assess the objectivity in the reading of the assay results, the interobserver agreement (using kappa statistics) between the first and second readers was measured. The ability of the assay to detect resistance to each drug separately as well as XDR TB (i.e., resistance to a fluoroquinolone and at least one of the following: amikacin, capreomycin, or kanamycin) was calculated for those strains that had corresponding MGIT SLD DST results. The ability of the test to detect resistance to EMB and interobserver agreement were calculated for all tested strains (MDR and non-MDR TB strains).

Statistical analysis was performed by use of Microsoft Excel and PASW Statistics (release 18; SPSS Inc., Chicago, IL) software.

Ethics statement. As no patient information was used in this study, ethics approval was not sought.

RESULTS

The interpretability of the Genotype MTBDR*sl* assay was high, varying between 98.0% and 100% for the first reading and between 95.5% and 100% for the second reading (Table 3).

The sensitivities for individual drugs (amikacin, kanamycin, capreomycin, and ethambutol) varied between 42.7% for kanamycin and 90.6% for capreomycin. The specificities for all these drugs were over 88%, with 100% specificity for amikacin and kanamycin. The PPV ranged from 90.0% (capreomycin) to 100% (amikacin and kanamycin); the NPV was lowest for ethambutol (50.2%) and highest for capreomycin (97.5%).

The sensitivities of the test for the fluoroquinolone group and the group of injectable drugs (AG-CP) were 92.3% and 41.7%, respectively; the sensitivity for XDR TB detection was 22.6%. The test showed almost 100% specificity and 100% PPV for these two groups of drugs as well as for XDR TB. The NPV ranged from 62.3% for injectable drugs to 93.9% for fluoroquinolones (Table 4).

TABLE 3 Interpretability of results obtained with the Genotype MTBDR*sl* assay across all sites[a]

Site	Reading	No. of positive results detected at site/total no. of positive samples (%) for SLD group			Mean interpretability (%)
		FQ	AG-CP	EMB	
1	1st	197/198 (99.5)	198/198 (100)	198/198 (100)	99.8
	2nd	193/198 (97.5)	195/198 (98.5)	197/198 (99.5)	98.5
2	1st	200/200 (100)	200/200 (100)	200/200 (100)	100
	2nd	199/200 (99.5)	200/200 (100)	200/200 (100)	99.8
3	1st	199/200 (99.5)	199/200 (99.5)	199/200 (99.5)	99.5
	2nd	193/200 (96.5)	193/200 (96.5)	191/200 (95.5)	96.2
4	1st	195/199 (98)	195/199 (98)	195/199 (98)	98
	2nd	195/198 (98.5)	195/198 (98.5)	193/198 (97.5)	98.2

[a] The mean interpretabilities for the first and second reads were 99.3% and 98.0%, respectively, for the fluoroquinolone (FQ) group; 99.4% and 98.4%, respectively, for the aminoglycoside-capreomycin (AG-CP) group; and 99.4% and 98.1%, respectively, for the ethambutol (EMB) group. SLD, second-line drugs.

The kappa values measuring interobserver agreement ranged between 0.91 and 1.00 ($P < 0.001$) for all tested drug groups. However, the kappa value was slightly lower (between 0.70 and 0.90) for the detection of XDR TB (Table 5).

Three sites made some mistakes in the interpretation of results (the proportion of wrongly interpreted strips did not exceed 3%).

DISCUSSION

The development and implementation of rapid molecular assays for the detection of resistance to reserve drugs are of vital importance in areas with high and medium TB burdens with high rates of MDR and XDR TB, including Russia and the Baltic States. The recently developed line probe assay for the detection of resistance to ethambutol, fluoroquinolones, and injectable drugs (Genotype MTBDR*sl*) has been evaluated in a few studies and demonstrated generally good performance characteristics; however, one study (9) reported suboptimal sensitivity for ethambutol and injectable drugs (especially kanamycin and capreomycin). The sensitivities varied significantly, probably indicating the presence of mutations in study isolate collections not recognized by the assay. The current multicenter international diagnostic accuracy study was aimed at assessing the performance and robustness of the assay with a large and diverse collection of TB isolates representing a variety of drug resistance profiles. Additionally, the ease of launching of the assay for use across new laboratories was tested.

Our results suggest that the Genotype MTBDR*sl* assay is relatively easy to introduce into routine settings; its performances were similar across multiple sites irrespective of whether the site had extensive, limited, or no previous experience with the test. Although bands indicating resistance to fluoroquinolones were the most difficult to read unambiguously, the interpretability of the Genotype MTBDR*sl* assay was very high at all sites. Interestingly, the interpretability was higher among the first readers than among the second readers, which might be explained by the more extensive experience of the second readers, who avoided unambiguous interpretations of fainter bands. This finding highlights the need for the standardization of assay readings and interpretations, perhaps through the implementation of automated reading systems; otherwise, extra training should be provided, and specific internal QC measures should be implemented.

In general, the assay is easy to interpret correctly: the interobserver agreement between two readers for fluoroquinolones, injectable drugs, ethambutol, and XDR was almost perfect at all four sites. Three sites made some mistakes in the interpretation of results, but the proportion of wrongly interpreted strips was negligible.

In the current study, the sensitivity of the assay for the detection of resistance was high for fluoroquinolones and lower for ethambutol (92.3% and 77.3%, respectively), which is consistent with most previous reports (4, 7). The assay demonstrated a good performance across all sites for the detection of resistance to amikacin and capreomycin, except for kanamycin. The absence of cross-resistance between kanamycin and amikacin in almost a quarter of the isolates (a phenomenon noted for Estonian strains and described previously by Kruuner et al. [11]) and the dominance of a specific clone of a Beijing family strain in Estonia (10, 12) harboring mutations not recognized by the assay might be the reasons for the extremely low ability of the assay to detect resistance to kanamycin. These observations are in agreement with a recent report from Vietnam, where the sensitivity of the assay was significantly affected by mutations in the *eis* gene, not recognized by the Genotype MTBDR*sl* assay and detected only by sequencing (9).

The inability of the assay to detect resistance to kanamycin in our study significantly affected its performance characteristics in regard to the detection of resistance to the group of injectable drugs and XDR TB. As the assay can be most useful in countries where the prevalence of MDR/XDR TB is high (where a similar situation regarding cross-resistance patterns and the dominance of specific genetic groups might be observed), it has to be further validated in these settings, and the inclusion of further probes for the detection of mutations in *eis* (in addition to *rrs*) genes should be considered. This is particularly useful since kanamycin is widely used for treatment in Eastern Europe and Central Asia, while it is arguably less commonly used elsewhere.

The demonstrated excellent specificity for key drugs makes this test extremely useful for screening, prompting the initiation of appropriate second-line therapy based on the assay results without waiting for traditional SLD DST results. The lower specificity for ethambutol might be misleading, as conventional phenotypic DST used as a gold standard in this study is itself problematic (13), potentially confounding the comparison.

TABLE 4 Performance of the Genotype MTBDR*sl* assay with strains tested at 4 sites compared to performance of MGIT 960 phenotypic DST[a]

Site	Individual drug or drug group	No. of concordant resistant results (MTBDR*sl*)/total no. of resistant isolates by MGIT + MTBDR*sl* (%)	No. of concordant sensitive results (MTBDR*sl*)/total no. of sensitive isolates by MGIT + MTBDR*sl* (%)	Total agreement (S + R) (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
1	FQ	69/81 (85.2)	92/104 (88.5)	93.1	90.8	94.8	93.2	92.9
	AMK	37/45 (82.2)	130/138 (94.2)	95.4	82.2	100.0	100.0	94.2
	KAN	37/86 (43.0)	89/138 (64.5)	72.0	43.0	100.0	100.0	64.5
	CAP	34/40 (85.0)	134/140 (95.7)	96.6	91.9	97.8	91.9	97.8
	AG-CP	37/89 (41.6)	87/139 (62.6)	70.5	41.6	100.0	100.0	62.6
	EMB	121/160 (75.6)	37/76 (48.7)	80.2	77.6	90.2	96.8	51.4
	XDR	8/37 (21.6)	134/163 (82.2)	83.0	21.6	100.0	100.0	82.2
2	FQ	71/82 (86.6)	94/102 (89.2)	93.6	92.2	94.8	93.4	93.8
	AMK	38/46 (82.6)	133/141 (94.3)	95.5	82.6	100.0	100.0	94.3
	KAN	38/88 (43.2)	91/141 (64.5)	72.1	43.2	100.0	100.0	64.5
	CAP	35/41 (85.4)	137/143 (95.8)	96.6	92.1	97.9	92.1	97.9
	AG-CP	37/89 (41.6)	85/137 (62.0)	70.1	41.6	100.0	100.0	62.0
	EMB	117/156 (75.0)	35/74 (47.3)	79.6	77.0	89.7	96.7	50.0
	XDR	9/39 (23.1)	135/165 (81.8)	82.8	23.1	100.0	100.0	81.8
3	FQ	75/86 (87.2)	93/104 (89.4)	93.9	93.8	93.9	92.6	94.9
	AMK	35/44 (79.6)	134/143 (93.7)	94.9	79.5	100.0	100.0	93.7
	KAN	35/86 (40.7)	93/143 (65.0)	71.9	41.2	100.0	100.0	65.0
	CAP	32/39 (82.1)	138/145 (95.2)	96.0	88.9	97.9	91.4	97.2
	AG-CP	38/92 (41.3)	89/143 (62.2)	70.2	41.3	100.0	100.0	62.2
	EMB	121/162 (74.7)	38/79 (48.1)	79.5	76.6	90.5	96.8	50.7
	XDR	9/40 (22.5)	139/170 (81.8)	82.7	23.1	99.3	90.0	82.2
4	FQ	73/84 (86.9)	91/102 (89.2)	93.7	92.4	94.8	93.6	93.8
	AMK	37/46 (80.4)	129/138 (93.5)	94.9	80.4	100.0	100.0	93.5
	KAN	37/85 (43.5)	90/138 (65.2)	72.6	43.5	100.0	100.0	65.2
	CAP	34/44 (77.3)	133/143 (93.0)	94.4	89.5	95.7	85.0	97.1
	AG-CP	38/90 (42.2)	86/138 (62.3)	70.5	42.2	100.0	100.0	62.3
	EMB	120/161 (74.5)	32/73 (43.8)	78.8	77.9	82.1	94.5	48.5
	XDR	9/40 (22.5)	135/166 (81.3)	82.3	22.5	100.0	100.0	81.3
Total (800 strains)	FQ	288/333 (86.5)	367/412 (89.1)	93.6	92.3	94.6	93.2	93.9
	AMK	147/181 (81.2)	526/560 (93.9)	95.2	81.2	100.0	100.0	93.9
	KAN	147/344 (42.7)	363/560 (64.8)	72.1	42.7	100.0	100.0	64.8
	CAP	135/164 (82.3)	542/571 (94.9)	95.9	90.6	97.3	90.0	97.5
	AG-CP	150/360 (41.7)	347/557 (62.3)	70.3	41.7	100.0	100.0	62.3
	EMB	479/639 (75.0)	142/302 (47.0)	79.5	77.3	88.2	96.2	50.2
	XDR	35/156 (22.4)	543/664 (81.5)	82.7	22.6	99.8	97.2	81.9

[a] FQs, fluoroquinolones; AMK, amikacin; KAN, kanamycin; CAP, capreomycin; AG-CP, aminoglycoside-capreomycin; EMB, ethambutol; XDR, extensive drug resistance; NPV, negative predictive value; PPV, positive predictive value; S, sensitive; R, resistant.

The high PPV confirms the applicability of the assay for MDR/XDR TB screening. However, whereas the NPV was very high for fluoroquinolones and ethambutol, it was low for AG-CP, again due to the low NPV for kanamycin (which might be of less significance outside Eastern Europe and Central Asia). The low sensitivity and low NPV for injectable drugs demonstrate that the current version of the assay cannot be used to rule out XDR TB, and phenotypic SLD DST remains necessary.

TABLE 5 Interobserver agreement across all field sites according to experience of the operators[a]

Site	Level of prior experience	Kappa value for individual drug group			
		FQs	AG-CP	EMB	XDR
1	Moderate	0.96	0.94	0.98	0.71
2	Extensive	0.93	0.92	0.91	0.70
3	None	0.94	1.00	0.99	0.90
4	None	0.93	0.93	0.89	0.74
All 4 sites		0.94	0.95	0.94	0.76

[a] The *P* value was <0.001 for all kappa values. FQs, fluoroquinolones; AG-CP, aminoglycoside-capreomycin; EMB, ethambutol; XDR, extensive drug resistance.

The current study is not free from limitations, and the study panel, containing only strains from a single geographical area characterized by a prevalence of highly conserved genotypes, could be considered one of these limitations. However, available published data show that genotypes similar to those prevalent in Estonia are widely spread across many countries of the former Soviet Union, including the Baltic States, Russia, and Central Asian countries (16, 20). Since all these areas have extremely high rates of drug resistance, we believe that our study results are important for the implementation of the assay in the given settings.

The study helped to establish an international platform of diagnostic trial field sites working according to common protocols. Future training needs when introducing new diagnostic assays into routine laboratory practice were identified and addressed.

The assay presents a good screening tool for the rapid detection of resistance to second-line anti-TB drugs; it can be recommended for use especially in countries with a high burden of MDR/XDR TB. For patients suspected of having MDR/XDR TB, the assay can be set up in parallel with DST for first-line drugs (FLDs) to avoid delays in the initiation of a suitable second-line therapeutic regimen.

One way of improving the assay performance would be to

modify the assay to better detect resistance to kanamycin (*eis*), which is particularly important in settings where only a partial cross-resistance between kanamycin and amikacin was observed. The ambiguity of the fluoroquinolone band intensity is another issue that could be addressed by the manufacturer.

Generally, in order to avoid interpretation errors, it would be useful to introduce a quality control of the readings (blinded rereading by a second person) or implement an automated reading system.

Further studies are needed to evaluate the performance characteristics of the assay in various settings with different strain collections and directly on patient specimens.

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